Week 4

June 27, 2016

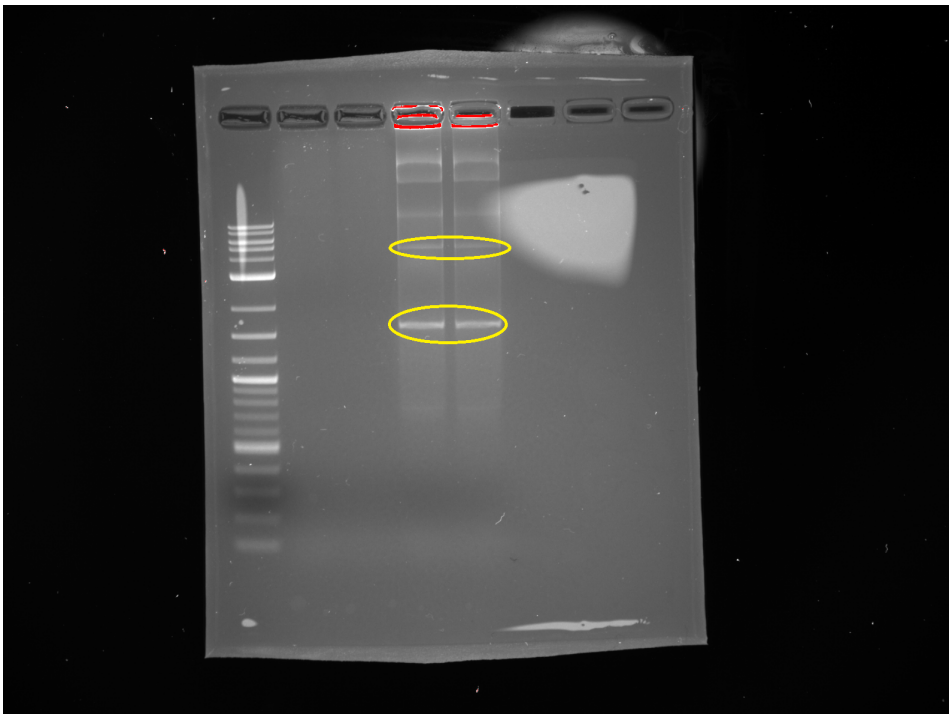
What we did today:

PCR
Liquid cultures
Drafted Email for Human Practices
Transformed RFP, InterLab Device 3, pRETRO-ON plasmids
Streaked GFP from Glycerol Stock
Made glycerol stock of pRETRO-ON

PCR:
Mixed two tubes of PCR reaction:
25 µL 2xPCR mastermix
1.25 µL dCas9 BamH1 primer
1.25 µL dCas9 Pac1 primer
22.5 µL DI water
1 picked colony of pdCas9

PCR protocol:
2 minutes at 95°C
----
30 seconds at 95°C
45 seconds at 50°C
4 minutes 30 seconds at 72°C
-------- repeat 30 times------
10 minutes at 72°C
Held at 10°C until taken out of PCR

Ran on Gel:



Cut out two pieces of gel circled above
Weighed each gel piece
2 µL of NT for every 1 µg of reaction
Allowed gel pieces to melt in 42°C water bath
Added to gel extraction columns
Spun for 1 minute
Dumped out flow through
Added 700 µL of NT3
Spun for 1 minute
Dumped out flow through
Spun for 2 minutes
Dumped out flow through
Put column into microfuge tube
Added 50 µL of NE
RT for 1 minute
Spun for 1 minute

Restriction:
50 µL of PCR extracted
6 µL cutsmart
2 µL Pac1
2 µL BamH1
Incubated in 37°C water bath over night

Transformations into DH10Beta cells

- GFP
- RFP
- pRetro-On
    - Made Glycerol Stocks of Each

PCR

PCR of dCas9

dCas9 grew better on 2x amp plates
Double digest of Pac1 and BamH1 on plasmid
10ug plasmid
5uL Cutsmart
2uL each enzyme
31uL water (50uL total)

Liquid cultures 3 tubes dCas9 in amp and LB

Gel wide wells (.6g agarose/60mL 1x TAE)
50uL sample + 10uL load dye + SYBR green
Lane 1: ladder
Lane 2: 30uL sample
Lane 3: 30uL sample

Cut out band with razor blade, place in tube and do tube kit

- Plasmid prep: pRetro
    Total = 50 µL
    - 5 µL CutSmart buffer
    - 10 µg pRetro
    - 2 µL each enzyme
    - 1 µL SAP after 2 hours
- Liquid culture:
    dCas9-humanized, 2X Amp LB (10 µL/tube), 3 tubes
- Gel
    pRetro: BamHI, PacI digest
- DNA purification from gel
    - 350 mg gel/100 mg * 200 µL NTI
    - Water bath to melt
    - Spin, add 600 µL NT3
    - 50 µL elution buffer

June 28, 2016

What We Did Today:
- Transformed pRETRO-ON
- Transformed RFP from iGEM 2016 competency pack
- Restreaked ptet-RFP, pLoe-RFP, and GFP
- PCR dCas9
- Emailed Mr. Sontheimer and Mr. Zhang about integrated human practices
  - (Sontheimer will not be in the office until July 18, email again around the 20?)

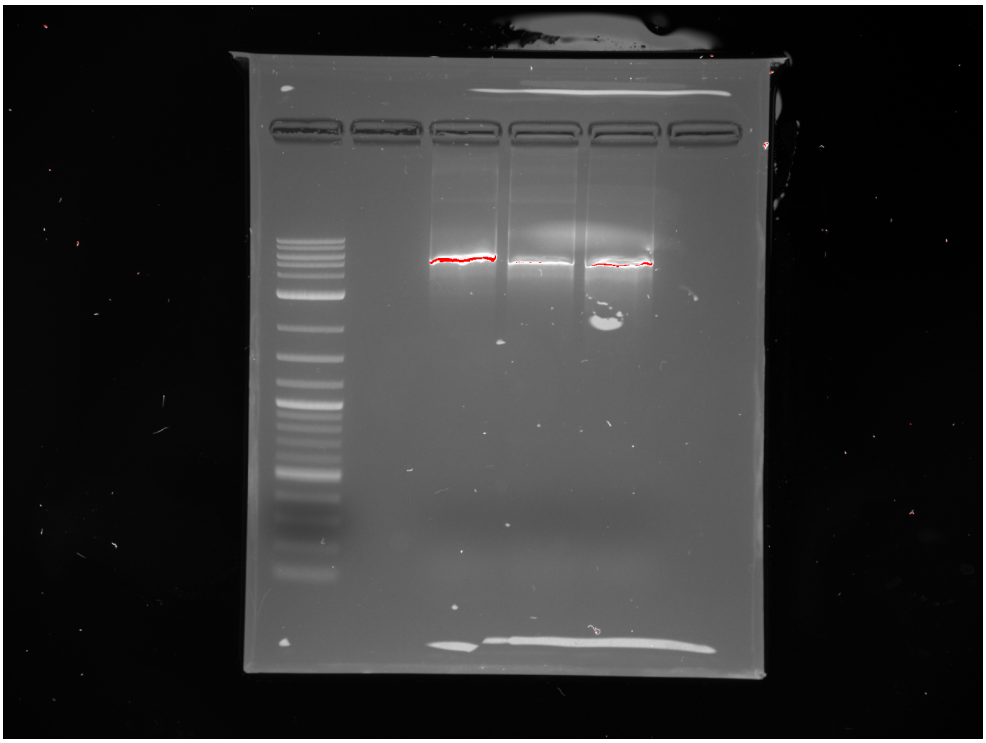
Heated each PCR reaction in 80°C water bath for 20 minutes

PCR Clean-up:
100 µL of NT and 50 µL of reaction into PCR clean-up columns
Spun for 1 minute
Dumped out flow through
Added 700 µL of NT3
Spun for 1 minute
Dumped out flow through
Spun for 2 minutes
Dumped out flow through
Put column into microfuge tube
Added 50 µL of NE
RT for 1 minute
Spun for 1 minute

Ligation:
Created 4 separate ligation reactions
1. 1 µL vector (pRetro-ON), 1 µL Insert (dCas9), 15 µL sterile water, 2 µL 10x ligation buffer, and 1 µL T4 ligase
2. 1 µL vector, 2.5 µL Insert, 13.5 µL sterile water, 2 µL 10x ligation buffer, and 1 µL T4 ligase
3. 1 µL vector, 7 µL Insert, 9 µL sterile water, 2 µL 10x ligation buffer, and 1 µL T4 ligase
4. 1 µL vector, 0 µL Insert, 16 µL sterile water, 2 µL 10x ligation buffer, and 1 µL T4 ligase (negative control)

Incubated at RT for 30 minutes
Transformed 5 µL of each ligation with 50 µL of DH10β cells
Plated onto 1xAMP plates
None grew

PCR
- Using dCas9 mini-preps
- 3 mini-preps:
  - 1.) 1.002 (diluted 1:8)
  - 2.) 0.897 (diluted 1:8)
  - 3.) 0.078



PCR Gel

Lane 1: Log2 Ladder
Lane 2: Empty
Lane 3: Mini-prep 1
Lane 4: Mini-prep 2
Lane 5: Mini-prep 3

Analysis: All of the bands for the three min-preps are at the same spot in the gel around the 6kb mark.

Cut out three pieces of gel where the bands were located above
Weighed each gel piece

1.) 0.57g -> 570mg -> 1140µL
2.) 0.46g -> 460mg -> 920µL
3.) 0.81g -> 810mg -> 1620µL

2 µL of NT for every 1 µg of reaction
Allowed gel pieces to melt in 42°C water bath
Added to gel extraction columns
Spun for 1 minute
Dumped out flow through
Added 700 µL of NT3
Spun for 1 minute
Dumped out flow through
Spun for 2 minutes

Dumped out flow through
Put column into microfuge tube
Added 50 µL of NE
RT for 1 minute
Spun for 1 minute

Restriction:
50 µL of PCR extracted
6 µL cutsmart
2 µL Pac1
2 µL BamH1
Incubated in 37°C water bath over night

Made glycerol stock of dCas9, in the #5 slot
Miniprepped dCas9 humanized

Digest confirmation of pgRNA
Digested withXho1, BamH1, EcoR1, Not1, and double digest of Not1 and EcoRi, and negative control

Ran gel
Lanes:
1: Ladder
2: Negative Control
3: Xho1
4: BamH1
5: EcoR1
6: Not1
7: Not1 EcoR1 double digest

Ligation of dCas9 into pRetro-ON
-4 types: 1 uL insert, 2.5 uL insert, and 7 uL insert
Incubated at RT for 30 min
Transformed into DH10Beta, (5uL ligation +50uL cells), plated on 1x AMP

- Miniprep: pgRNA-humanized (3 samples)
    - 1: 100.7 ng
    - 2: 107.7 ng
    - 3: 833.4 ng
- Digest & Gel
    - pgRNA-humanized (used 833.4 ng stock, 1.2 µL/sample)
    - Lanes (L to R): Ladder, Negative, XhoI, BamHI, ECoRI, NotI, ECoRI+NotI
    - 12 µL/well, 4 µL dye/tube

June 29, 2016

What We Did Today:
- PCR Cleanup
- Transformed ligations from digest (using both DH5(alpha) cells and DH10(Beta) cells
- Made liquid cultures of RFP and GFP
- Streaked and made liquid culture of new DH5(alpha) cells

Results from yesterday's transformations:
- RFP from cell competency pack worked
- PRETRO-ON was overgrown (possible problem with competent cells)
- Restreaks all worked

PCR Clean-up
- 100 µL of NT and 50 µL of reaction into PCR clean-up columns
- Spun for 1 minute
- Dumped out flow through
- Added 700 µL of NT3
- Spun for 1 minute
- Dumped out flow through
- Spun for 2 minutes
- Dumped out flow through
- Put column into microfuge tube
- Added 50 µL of NE
- RT for 1 minute
- Spun for 1 minute

Ligation:
- Using protocol
- Made different dilution (neg control, 1 µL, 2.5 µL, and 7µL)
- Transformed using both Dh5(alpha) and DH10(beta) cells

Made liquid cultures of P-Tag RFP

Transformation of ligation reaction into EGM2K lambda cells.
7.5uL of reaction into 50uL cells, shaker for 2 hours plated on 1x AMP plates

New insert
-80 C 20min and let cool to rt,
PCR clean up. Ligation
Transformed into DH10Beta and DH5Alpha cells

• Transformation
- Cells: DH5α, EMG2Kλ
- DNA: ligation products (pRetro-dCas9), 1, 2.5, 7 µL samples

- 7.5 µL DNA/tube
- DH5α in 1X Amp, EMG2Kλ in 2X Amp

• PCR set up

dCas9, 1.002 µg/µL dilute 1:8

- 22.5 µL dH_2O
- 1 µL DNA
- 1.25 (1.3) µL 3' (PacI) & 5' (BamHI) primer each
- 25 µL 2X PCR Mastermix
- PCR cycle * 30

June 30, 2016

What we did today:

Checked cultures – RFP did not glow red, GFP did

Unable to make glycerol stocks because didn't glow red **(USE CHLOR NEXT TIME NOT AMP!!)**

Results from Transformations:

Transformations using DH5(alpha) cells were very overgrown

Transformations using DH10(beta) barely grew

DH5a Competent Cell Protocol:

1.) 2ml of the liquid culture into flask with 200mL of LB (no antibiotics)
2.) Shakubator to grow for 1.5hrs
3.) Incubate until OD600nm of 0.3. Continue incubation until OD reading is correct
4.) Pre-chill 4 50 mL connicle tubes and large centrifuge to 4 degrees.
5.) Transfer 20mL of cells to each tube
6.) Incubate on ice for 20 mins.
7.) Combine two tubes into one (results in two tubes with 40mL each)
8.) Centrifuge at 4 degrees at 3000g for 15 Mins
9.) Pour out supernatant into waste.
10.) Resuspend pellet in 5mL of ice cold CCMB80 buffer
11.) Incubate for 20 mins on ice
12.) Put 200µL into chilled microcentrifuge tubes
13.) Store at –80
14.) Test Competency by transforming with GFP.

PCR run in gel
5uL PCR rxn + 1uL dye
Lane 1: Ladder
Lane 2: -
Lane 3: PCR rxn 1
Lane 4: -
Lane 5: PCR rxn 2
Lane 6: -
Lane 7: PCR rxn 3
Lane 8: -
Lane 9: PCR rxn 4
All had bands except #4, 1 the brightest
45uL PCR cleanup
90uL NT1
spun in yellow column
add 700uL NT3 spin 1 min
dump spin 2 min
add 50uL elution buffer

digestion 50uL of above
6uL smartcut buffer
2uL Pac1
2uL BamH1
digested 1.5 hours
Heat kill 20 mins 80 degrees
Ran another gel
#1 & 3 had bands

Ligation:
EMG2k lambda cells 2xamp plates

Insert: 0, 2, 7, 15uL
water: 15, 13, 8, 0uL
2uL vector
2uL ligase buffer
1uL ligase

sits room temp 30 mins
Transform 5uL into 50uL cells
Transformation protocol
Wait full 2 hours on the shaker

APOBEC1 PCR

- Gel
  - PCR product (dCas9)
    - 5 µL/well + 1 µL dye
  - Lanes (L to R): Ladder, 1, 2, 3, 4
- PCR clean up
  - PCR product (dCas9)
    - ~45 µL PCR + 200 µL NT1/100 µL PCR = 90 µL NT1
    - 700 µL NT3
    - 50 µL elution buffer
- Digest
  - PacI & BamHI
  - 60 µL final volume
- Gel
  - Digested PCR clean up product (dCas9)
    - 5 µL/well + 1 µL dye
  - Lanes (L to R): Ladder, 1, 2, 3, 4
- Heat kill enzyme in the rest of the digest (80 ˚C, 20 min)
- Ligation
  - Vector: pRetro
  - Insert: PCR product (dCas9)
    - Water: 15, 13, 8, 0 µL
    - Insert: 0, 2, 7, 15 µL

- 2 μL buffer
- 2 μL vector
- 1 μL ligase
- 30 min in RT

• Transformation

Cells: EMG2Kλ

- 50 μL cell
- 5 μL ligation product
- 2X Amp plates